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1 **Development and application of environmental DNA surveillance**
2 **for the threatened crucian carp (*Carassius carassius*)**

3

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25

26 **Abstract**

27

28 **1.** The crucian carp (*Carassius carassius*) is one of few fish species associated with small ponds in
29 the UK. These populations contain genetic diversity not found in Europe and are important to
30 conservation efforts for the species, which has declined across its range in Europe. Detection and
31 monitoring of extant crucian carp populations are crucial for conservation success. Environmental
32 DNA (eDNA) analysis could be very useful in this respect as a rapid, cost-efficient monitoring
33 tool.

34 **2.** We developed a species-specific quantitative PCR (qPCR) assay for eDNA surveillance of
35 crucian carp to enable non-invasive, large-scale distribution monitoring. We compared fyke
36 netting and eDNA at ponds with (N = 10) and without (N = 10) crucian carp for presence-absence
37 detection. We examined biotic (crucian carp density represented by catch-per-unit-effort estimate
38 - CPUE) and abiotic influences on eDNA detection probability using a hierarchical occupancy
39 model, and eDNA quantification using a mixed-effects model.

40 **3.** eDNA analysis achieved 90% detection for crucian carp (N = 10), failing in only one pond where
41 presence was known. CPUE estimate and conductivity had positive and negative influences on
42 eDNA detection probability in qPCR replicates respectively. Similarly, conductivity had a
43 negative effect on DNA copy number, whereas copy number increased with CPUE estimate.

44 **4.** Our results demonstrate that eDNA could enable detection of crucian carp populations in ponds
45 and benefit ongoing conservation efforts, but imperfect species detection in relation to biotic and
46 abiotic factors and eDNA workflow requires further investigation. Nonetheless, we have
47 established an eDNA framework for crucian carp as well as sources of imperfect detection which
48 future investigations can build upon.

49

50 **1. Introduction**

51

52 The crucian carp (*Carassius carassius*) (Figure 1) is an elusive, benthic fish species popular
53 with anglers (Copp, Warrington & Wesley, 2008b; Sayer et al., 2011). As one of few fish
54 associated with small ponds, this species may have an important ecological role but its
55 relationship with other lentic biodiversity is understudied (Copp & Sayer, 2010; Stefanoudis
56 et al., 2017). Although listed as ‘Least Concern’ on the International Union for Conservation
57 of Nature (IUCN) Red List of Threatened Species, the species has declined throughout its
58 native range of Northwest and Central Europe (Copp et al., 2008b; Sayer et al., 2011), with
59 local extinctions across the UK (Copp & Sayer, 2010). The county of Norfolk in eastern
60 England was believed to hold abundant and widely distributed crucian carp populations, but
61 research indicates heavy (~75%) declines in this region (Sayer et al., 2011). Declines of the
62 crucian carp throughout its range are due to habitat loss (Copp et al., 2008b; Sayer et al.,
63 2011), species displacement by the invasive gibel carp (*Carassius gibelio*) (Copp et al.,
64 2008b; Tarkan et al., 2009; Sayer et al., 2011), and genetic introgression through
65 hybridisation (Hänfling et al., 2005). Indeed, Sayer et al. (2011) observed only 50% of
66 crucian carp ponds to be uninhabited by goldfish (*Carassius auratus*), common carp
67 (*Cyprinus carpio*), or their hybrids with crucian carp.

68 Prior to the 1970s, crucian carp were thought to have been introduced to the UK
69 alongside common carp and were classed as non-native (Maitland, 1972). Wheeler (1977)
70 deemed the species native to southeast England based on archaeological evidence and a
71 historic distribution that mirrored native cyprinids. Conservation organisations (e.g. English
72 Nature, Environment Agency) later recognised the crucian carp as native and threatened
73 (Smith & Moss, 1994; Environment Agency, 2003), but recent genetic evidence supports
74 anthropogenic introduction of the crucian carp to the UK during the 15th century (Jeffries et

75 al., 2017). Nonetheless, many introduced species in the UK are now naturalised, and several
76 provide ecological and economical benefits (Manchester & Bullock, 2000). Evidence
77 suggests that the crucian carp is characteristic of small, plant-dominated, high-quality ponds
78 (Copp et al., 2008b; Sayer et al., 2011; Stefanoudis et al., 2017), and English populations
79 contain a substantial proportion of the overall genetic diversity for the species across Europe.
80 English crucian carp populations may buffer species displacement by gibel carp at the
81 European level (Jeffries et al., 2017), but are threatened by hybridisation with goldfish and
82 possible displacement (Hänfling et al., 2005; Tarkan et al., 2009) as well as anthropogenic
83 activity (Copp, Černý & Kováč, 2008a).

84 In 2010, the crucian carp was designated as a Biodiversity Action Plan (BAP) species
85 in the county of Norfolk (Copp & Sayer, 2010; Sayer et al., 2011). To meet the BAP aims,
86 local conservation efforts have included species reintroduction, pond restoration, and
87 eradication of goldfish (Sayer et al., 2011). However, current distribution records are
88 unreliable as individuals are frequently misidentified as the feral brown variety of goldfish
89 due to high physical similarity (Copp et al., 2008a; Tarkan et al., 2009), and many pond
90 populations are mixtures of true crucian carp and crucian carp x goldfish hybrids (Hänfling et
91 al., 2005). Consequently, distribution maps have been called into question and further
92 monitoring is needed to ensure long-term success of established and reintroduced crucian
93 carp populations (Copp et al., 2008a; Tarkan et al., 2009).

94 Primarily, crucian carp are surveyed using fyke netting or electrofishing, but these
95 methods can be costly and time-consuming. Environmental DNA (eDNA) analysis offers a
96 potentially rapid and cost-effective approach to fish monitoring (Jerde et al., 2011; Sigsgaard
97 et al., 2015; Wilcox et al., 2016; Hänfling et al., 2016; Hinlo et al., 2017a). Species are
98 identified using DNA deposited in the environment by individuals via secretions, excretions,
99 gametes, blood, or decomposition (Lawson Handley, 2015). eDNA has been applied

100 worldwide to survey for invasive freshwater fish (Jerde et al., 2011; Keskin, 2014; Robson et
101 al., 2016; Hinlo et al., 2017a), and is now used routinely to monitor Asian carp
102 (*Hypophthalmichthys* spp.) invasion in the Great Lakes, USA (Farrington et al., 2015). A
103 quantitative PCR (qPCR) assay targeting crucian carp was also published in the context of
104 early warning invasion monitoring for fish species that may arrive in Canada (Roy et al.,
105 2017), but was only tested on tissue-derived DNA. Of equal importance to invasion
106 monitoring, eDNA analysis has enhanced surveys for threatened and endangered freshwater
107 fish (Sigsgaard et al., 2015; Schmelzle & Kinziger, 2016; Piggott, 2016; Bylemans et al.,
108 2017).

109 eDNA analysis has been conducted with conventional PCR (PCR) (Ficetola et al.,
110 2008; Jerde et al., 2011), but qPCR and droplet digital PCR (ddPCR) are suggested to
111 perform better, suffer less from inhibition, and enable abundance or biomass estimation
112 (Nathan et al., 2014). However, these estimates can be inconsistent across habitats and target
113 organisms. In flowing water, Hinlo et al. (2017a) found no relationship between DNA copy
114 number and conventional density estimates of common carp, yet Takahara et al. (2012)
115 observed a positive association between common carp biomass and eDNA concentration in
116 ponds. Environmental variables play a substantial role in abundance/biomass estimation by
117 influencing the ecology of eDNA (Barnes et al., 2014). Variables examined have included
118 temperature, pH, salinity, conductivity, anoxia, sediment type, and UV light (Takahara et al.,
119 2012; Barnes et al., 2014; Pilliod et al., 2014; Keskin, 2014; Strickler, Fremier & Goldberg,
120 2015; Robson et al., 2016; Buxton et al., 2017b; Buxton, Groombridge & Griffiths, 2017a;
121 Weltz et al., 2017; Stoeckle et al., 2017; Goldberg, Strickler & Fremier, 2018). However,
122 these variables are not always measured and only a handful of studies have assessed their
123 effects in ponds (Takahara et al., 2012; Buxton et al., 2017a, b; Goldberg et al., 2018).

124 In this study, we developed a species-specific qPCR assay for the threatened crucian

125 carp. We evaluated presence-absence detection with eDNA compared to fyke netting, and
126 investigated the influence of biotic and abiotic factors on eDNA detection and quantification.
127 We hypothesised that: (1) eDNA and fyke netting would provide comparable presence-
128 absence records for crucian carp, and (2) eDNA detection and quantification would be
129 influenced by crucian carp density, temperature, pH, conductivity, surface dissolved oxygen,
130 macrophyte cover, and tree shading. We provide an eDNA framework for crucian carp
131 monitoring which holds promise for routine survey.

132

133

134

135 **2. Methods**

136

137 **2.1 Study sites**

138

139 We studied 10 ponds with confirmed crucian carp presence at different densities and 10
140 fishless ponds in Norfolk (Figure 2). This region is low-lying (<100 m above sea level) and
141 mainly agricultural. All study ponds were selected to be small (<40 m in max. dimension),
142 shallow (<2 m), macrophyte-dominated, with a largely open-canopy and thus minimal
143 shading of the water surface. Ponds were largely surrounded by arable fields, excluding one
144 located in woodland. No specific permits were required for sampling but relevant landowner
145 permissions were obtained.

146

147

148 **2.2. Conventional survey**

149

150 Crucian carp presence-absence was confirmed at each pond by fyke netting between 2010
151 and 2016. Bar two ponds surveyed in 2013 and 2015, all crucian carp ponds were last
152 surveyed in 2016. Where possible, double-ended fyke nets were set perpendicular to the bank
153 or to beds of aquatic vegetation and exposed overnight (for c. 16 h), with the number of fyke
154 nets set being proportional to pond size. This provided CPUE estimates of relative densities,
155 which are the number of fish captured per fyke net per 16 h exposure. Environmental data
156 were collected between May and August from 2010 to 2017. Conductivity, pH, surface
157 dissolved oxygen, and water temperature were measured with a HACH HQ30d meter (Hach
158 Company, CO, USA), and alkalinity was determined by sulphuric-acid titration using a
159 HACH AL-DT kit (Hach Company, CO, USA). Percentages of macrophyte cover and
160 shading of ponds by trees and scrub were estimated visually.

161

162

163 **2.3 eDNA sampling, capture and extraction**

164

165 Five 2 L surface water samples were collected from the shoreline of each pond using sterile
166 Gosselin™ HDPE plastic bottles (Fisher Scientific UK Ltd, UK) and disposable gloves.
167 Samples were taken at equidistant points around the pond perimeter where access permitted.
168 All ponds without crucian carp were sampled on 22nd August 2016. Water samples were
169 transported on ice in sterile coolboxes to the Centre for Ecology and Hydrology (CEH),
170 Wallingford, stored at 4 °C, and vacuum-filtered within 24 hours of collection. Coolboxes
171 were sterilised using 10% v/v chlorine-based commercial bleach (Elliott Hygiene Ltd, UK)
172 solution and 70% v/v ethanol solution before ponds containing crucian carp were sampled on
173 25th August 2016. Samples were handled in the same way as those from fishless ponds. For

174 each pond, a full process blank (1 L molecular grade water) was taken into the field and
175 stored in coolboxes with samples. Blanks were filtered and extracted alongside pond samples
176 to identify contamination.

177 Where possible, the full 2 L of each sample was vacuum-filtered through sterile 0.45
178 μm cellulose nitrate membrane filters with pads (47 mm diameter; Whatman, GE Healthcare,
179 UK) using Nalgene filtration units. One hour was allowed for each sample to filter but if
180 filters clogged during this time, a second filter was used. After 2 L had been filtered or one
181 hour had passed, filters were removed from pads using sterile tweezers and placed in sterile
182 47 mm petri dishes (Fisher Scientific UK Ltd, UK), sealed with parafilm (Sigma-Aldrich[®],
183 UK), and stored at -20 °C. The total volume of water filtered and the number of filters used
184 per sample were recorded for downstream analysis (Table S1). After each round of filtration
185 (samples and blanks from two ponds), all equipment was sterilised in 10% v/v chlorine-based
186 commercial bleach (Elliott Hygiene Ltd, UK) solution for 10 minutes, immersed in 5% v/v
187 MicroSol detergent (Anachem, UK), and rinsed with purified water.

188 All filters were transported on ice in a sterile coolbox to the University of Hull and
189 stored at -20 °C until DNA extraction one week later. DNA was isolated from filters using
190 the PowerWater[®] DNA Isolation Kit (MO BIO Laboratories, CA, USA) following the
191 manufacturer's protocol in a dedicated eDNA facility at the University of Hull, devoted to
192 pre-PCR processes with separate rooms for filtration, DNA extraction, and PCR preparation
193 of environmental samples. Duplicate filters from the same sample were co-extracted by
194 placing both filters in a single tube for bead milling. Eluted DNA (100 μL) concentration was
195 quantified on a Qubit[™] 3.0 fluorometer using a Qubit[™] dsDNA HS Assay Kit (Invitrogen,
196 UK). DNA extracts were stored at -20 °C until further analysis.

197

198

199 2.4 Assay design, specificity and sensitivity

200

201 We designed a novel qPCR assay to target a 118 bp amplicon (73 bp excluding primers)
202 within the mitochondrial cytochrome *b* (*cytb*) gene, specific to crucian carp. Crucian carp
203 sequences from Jeffries et al. (2016) were aligned using MAFFT in AliView (Larsson, 2014)
204 to sequences downloaded from the NCBI nucleotide (nt) database for 23 closely related
205 species of European freshwater fish (Table S2), and a consensus sequence for each species
206 was identified (Figure 3). Sequences were visually compared to maximise nucleotide
207 mismatches between crucian carp and non-target species, particularly goldfish and common
208 carp, and minimise theoretical risk of non-specific amplification. Mismatches in primer
209 regions were maximised over the probe region to increase specificity (Wilcox et al., 2013).
210 Species-specific primers CruCarp_CytB_984F (5'-AGTTGCAGATATGGCTATCTTAA-3')
211 and CruCarp_CytB_1101R (5'-TGGAAAGAGGACAAGGAATAAT-3'), and corresponding
212 probe CruCarp_CytB_1008Probe (FAM 5'-
213 ATGGATTGGAGGCATACCAGTAGAACACC-3' BHQ1) were selected on this basis.

214 Primers without probe were tested *in silico* using ecoPCR (Ficetola et al., 2010)
215 against a custom, phylogenetically curated reference database that was constructed for eDNA
216 metabarcoding of lake fish communities in Windermere, Lake District National Park,
217 England, which contains 67 freshwater fish species confirmed or potentially present in the
218 UK (Hänfling et al., 2016). Parameters set allowed a 50-150 bp fragment and maximum of
219 three mismatches between each primer and each sequence in the reference database.
220 Specificity of primers (without probe) was also tested against the full NCBI nucleotide (nt)
221 database using Primer-BLAST (Ye et al., 2012) with default settings.

222 The primers were tested with PCR, following which primer and probe concentrations,
223 standard curve preparation, and cycling conditions for qPCR were optimised (Supporting

224 Information: Appendix 1). All subsequent qPCR analyses were performed using the
225 conditions detailed in section 2.5. Primers and probe were validated *in vitro* using tissue
226 DNA (standardised to 1 ng/ μ L) from fin clips of 10 non-target species (1 UK individual per
227 species) related to crucian carp (Table S3, Figures S1-3). The positive control and No
228 Template Control (NTC) were crucian carp DNA and molecular grade water (Fisher
229 Scientific UK Ltd, UK) respectively. The limits of detection (LOD, the lowest concentration
230 where at least one technical replicate amplified crucian carp DNA) and quantification (LOQ,
231 the concentration at which all technical replicates consistently amplified crucian carp DNA)
232 (Agersnap et al., 2017) were established using the qPCR standards (10^6 to 1 copy/ μ L) (Figure
233 S4). Five technical replicates were performed for standards, controls, and samples in tests of
234 assay specificity and sensitivity.

235

236

237 **2.5 Detection and quantification of crucian carp eDNA**

238

239 All qPCR reactions were prepared in a UV and bleach (Elliott Hygiene Ltd, UK) sterilised
240 laminar flow hood in the dedicated eDNA facility at the University of Hull. Reactions were
241 performed in a total volume of 20 μ L, consisting of 2 μ L of template DNA, 1 μ L of each
242 primer (Forward 900 nM, Reverse 600 nM), 1 μ L of probe (125 nM) (Integrated DNA
243 Technologies, Belgium), 10 μ L of TaqMan[®] Environmental Master Mix 2.0 (Life
244 Technologies, CA, USA), and 5 μ L molecular grade water (Fisher Scientific UK Ltd, UK).
245 Once eDNA samples and three NTCs were added to each 96-well plate, the plate was sealed
246 and transported to a separate laboratory on a different floor for addition of the standard curve
247 and three positive controls (crucian carp DNA, 0.01 ng/ μ L) in a UV and bleach sterilised
248 laminar flow hood.

249 Our standard curve was a synthesised 500 bp gBlocks[®] Gene Fragment (Integrated
250 DNA Technologies, Belgium) based on GenBank accessions (KT630374 - KT630380) for
251 crucian carp from Norfolk (Jeffries et al., 2016). Copy number for the gBlocks[®] fragment
252 was estimated by multiplying Avogadro's number by the number of moles. We performed a
253 10-fold serial dilution of the gBlocks[®] fragment to generate a 6-point standard curve that
254 ranged from 10⁶ to 10 copies/μL. eDNA samples were compared to these known
255 concentrations for quantification (Hinlo et al., 2017a). Each standard was replicated five
256 times on each qPCR plate. Similarly, five technical replicates were performed for every
257 sample and full process blank from each pond.

258 After addition of standards and positive controls, plates were again sealed and
259 transported to a separate laboratory on a different floor where qPCR was conducted on a
260 StepOnePlus™ Real-Time PCR system (Life Technologies, CA, USA). Thermocycling
261 conditions consisted of incubation for 5 min at 50 °C, a 10 min denaturation step at 95 °C,
262 followed by 60 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. We
263 used 60 cycles for consistency with optimisation tests, but cycling could be reduced to 45
264 cycles for subsequent applications (see Supporting Information: Appendix 1). A small-scale
265 comparison of eDNA detection and concentration using PCR and qPCR was also conducted
266 (Supporting Information: Appendix 1).

267 Amplifications were considered positive detections if the exponential phase occurred
268 within 45 reaction cycles as the mean C_q value was 40.07 for the LOD (1 copy/μL). A pond
269 was considered positive for crucian carp if two or more of the five technical replicates from a
270 sample returned positive, or more than one sample returned any positive technical replicates
271 (Goldberg et al., 2016). False negatives were obtained for one pond, therefore all samples
272 were tested for inhibition by spiking duplicate qPCR reactions with a known concentration of
273 synthetic crucian carp template (1000 copies/μL) (Jane et al., 2015).

274

275

276 **2.6 DNA sequencing**

277

278 Non-target DNA extracts and full-process blanks that amplified with qPCR were Sanger
279 sequenced alongside a representative eDNA sample from each positive pond (N = 9) to
280 confirm sequence identity. Purification and sequencing was performed by Macrogen Europe
281 (Amsterdam, The Netherlands) in triplicate in the forward direction. Sequences were edited
282 using CodonCode Aligner (CodonCode Corporation, MA, USA) with default settings.
283 Sequences were then manually aligned in AliView (Larsson, 2014) and poor quality
284 sequences were discarded (Figure S5). Primers were removed from remaining sequences, and
285 sequences identified against the full NCBI nucleotide (nt) database using the NCBI BLASTn
286 tool.

287

288

289 **2.7 Data analysis**

290

291 Technical replicates for each qPCR standard that differed by $>0.5 C_q$ from the average of the
292 five technical replicates performed were discarded to minimise bias induced by pipetting
293 error. All technical replicates for eDNA samples were retained, and those which failed to
294 amplify were classed as 0 copies/ μL (Goldberg et al., 2016). The C_q values for each set of
295 technical replicates were averaged and quantified to provide a single DNA copy number for
296 each sample. Samples with no positive amplifications were assigned a DNA copy number of
297 zero. DNA copy numbers of samples were then averaged to generate a single DNA copy
298 number for each pond.

299 All subsequent data analyses were performed in the statistical programming
300 environment R v.3.4.2 (R Core Team, 2017). Effects of water volume filtered, number of
301 filters used, and water sample content on DNA copy number of samples were tested and
302 reported in Supporting Information (see Appendices 1, 2; Figures S6, S7). Results and
303 discussion of the PCR-qPCR comparison are also reported in Supporting Information
304 (Appendices 2-3; Table S4; Figure S8). The R package ‘eDNAoccupancy’ v0.2.0 (Dorazio &
305 Erickson, 2017) was used to fit a Bayesian, multi-scale occupancy model to estimate eDNA
306 detection probability at sites where crucian carp were confirmed as present by fyke netting.
307 Existing eDNA literature was used to identify biotic and abiotic factors reported to affect
308 eDNA detection, persistence and degradation, and construct hypotheses regarding their
309 effects on eDNA detection probability in water samples (θ), and eDNA detection probability
310 in qPCR replicates (p). No covariates were included at the site level (ψ) as ponds were
311 occupied by crucian carp and eDNA should have been present. At the sample level, more
312 individuals (reflected by CPUE) should increase eDNA concentration and improve detection.
313 Temperature can increase physical, metabolic, or behavioural activity of organisms resulting
314 in more eDNA release, breakdown, and degradation (Takahara et al., 2012; Pilliod et al.,
315 2014; Strickler et al., 2015; Robson et al., 2016; Lacoursière-Roussel, Rosabal & Bernatchez,
316 2016; Buxton et al., 2017b; Bylemans et al., 2017). Links established between eDNA and pH
317 support greater detectability, concentration, and persistence of eDNA in more alkaline waters
318 (Barnes et al., 2014; Strickler et al., 2015; Goldberg et al., 2018). Conductivity relates to
319 Total Dissolved Solids (TDS) and sediment type, which can impair eDNA detection due to
320 release of inhibitory substances and their capacity to bind DNA (Buxton et al., 2017a;
321 Stoeckle et al., 2017). Vegetated ponds reduce UV exposure thereby preserving eDNA
322 (Barnes et al., 2014), and are susceptible to terrestrialisation which can create anoxic
323 conditions that may slow eDNA degradation (Barnes et al., 2014; Pilliod et al., 2014; Weltz

324 et al., 2017). At the qPCR replicate level, covariates again included CPUE as higher eDNA
325 concentration should improve amplification success and consistency, whereas conductivity
326 may indicate inhibitory substances that cause amplification failure.

327 Prior to modeling, all environmental variables were assessed for collinearity using
328 Spearman's correlation coefficient and Variance Inflation Factors (VIFs) calculated using the
329 R package 'car' v2.1-6 (Fox & Weisberg, 2011). Variables were considered collinear and
330 removed if $r > 0.3$ and $VIF > 3$ (Zuur et al., 2009), following which candidate variables (CPUE,
331 conductivity, pH, and percentage of macrophyte cover) were centred and scaled to have a
332 mean of 0 and standard deviation of 1. We constructed 64 models which assumed a constant
333 probability of eDNA occurrence at the site level, and different covariate combinations at the
334 sample and qPCR replicate levels. Models were ranked (Table S5) according to posterior
335 predictive loss criterion (PPLC) under squared-error loss and the widely applicable
336 information criterion (WAIC). The model with the best support was selected for comparison
337 to the null model without covariates at the entire sampling hierarchy.

338 We examined the influence of biotic and abiotic factors on eDNA quantification using
339 a generalised linear mixed effects model (GLMM) within the R package 'glmmTMB' v0.2.0
340 (Brooks et al., 2017). Collinearity was assessed as above, leaving CPUE, pH, conductivity,
341 and percentage of macrophyte cover as explanatory variables. Pond was modeled as a random
342 effect to account for spatial autocorrelation in our data set and the influence of other
343 properties inherent to each pond, whereas all other explanatory variables were fixed effects.
344 A Poisson distribution was specified as the nature of the response variable (DNA copy
345 number) was integer count data. Validation checks were performed to ensure all model
346 assumptions were met and absence of overdispersion (Zuur et al., 2009). Model fit was
347 assessed visually and with the Hosmer and Lemeshow Goodness of Fit Test (Hosmer &
348 Lemeshow, 2000) using the R package 'ResourceSelection' v0.3-0 (Lele et al., 2014). Model

349 predictions were obtained using the predict() function and upper and lower 95% CIs were
350 calculated from the standard error of the predictions. All values were bound in a new data
351 frame and model results plotted for evaluation using the R package ‘ggplot2’ v2.2.1
352 (Wickham, 2009). All R scripts and corresponding data have been deposited in a dedicated
353 GitHub repository (https://github.com/lrharper1/crucian_carp_eDNA_surveillance), which
354 has been permanently archived (<https://doi.org/10.5281/zenodo.1421602>).

355

356

357

358 **3. Results**

359

360 **3.1 Assay specificity and sensitivity**

361

362 Only crucian carp amplified in ecoPCR, confirming primer specificity. Non-target species
363 returned by primer-BLAST against the full NCBI nucleotide (nt) database were *Barilius*
364 *bakeri* (a Cyprinid fish restricted to India, 6 mismatches), *Naumovozyma dairensis* (fungi, 8
365 mismatches), and *Medicago trunculata* (plant, 8 mismatches). Our probe sequence could not
366 be included *in silico* but would likely increase specificity. Tissue extracts from common rudd
367 (*Scardinius erythrophthalmus*) and European chub (*Squalius cephalus*) included in qPCR
368 assay specificity tests were amplified by primers and probe, but possessed low DNA copy
369 number (<10 copies/μL). In a later test, common carp DNA also amplified (<10 copies/μL).
370 However, no amplification was observed for NTCs, fresh tissue extracts obtained from rudd
371 and chub, or eDNA samples from locations where crucian carp were absent and these species
372 were present (data not shown). DNA sequencing confirmed cross-contamination of reference

373 material, where sequences were either identified as crucian carp or of poor quality (Table 2).
374 Our assay was highly sensitive with a LOD of 1 copy/ μ L and LOQ of 10 copies/ μ L.

375

376

377 **3.2 qPCR analysis**

378

379 The qPCR assay had an average amplification efficiency of 93.61% (range 79.61-102.49%)
380 and an average R^2 value of 0.998 (range 0.995-0.999) for the standard curve. Only one plate
381 had a qPCR efficiency lower than 90% but the standard curve quantified as expected, thus
382 qPCR was not repeated. No amplification occurred in NTCs, but the full process blank for
383 one pond (POFA4) amplified (<10 copies/ μ L). This was the only contaminated blank as the
384 blank for pond POHI filtered alongside POFA4 and POHI samples, and blanks downstream
385 of these samples did not amplify. Partial inhibition (<1000 copies/ μ L) occurred in a single
386 sample from four different ponds: PYES2 (no crucian carp), RAIL, POHI, and GUES1
387 (crucian carp present). However, partially inhibited samples all possessed >0 copies/ μ L when
388 originally tested, and copy number did not differ substantially (higher in one instance) from
389 other samples belonging to the same pond (Table S1). Consequently, partial inhibition did not
390 influence detectability in our study, and problematic samples were not treated for inhibition
391 and qPCRs were not repeated.

392

393

394 **3.3 Presence-absence detection**

395

396 eDNA surveillance detected crucian carp in 90% of the study ponds (N = 10) with confirmed
397 presence. Sanger sequencing of representative samples confirmed species identity as crucian

398 carp (Table 2). eDNA failed entirely in one pond (CHIP) that contained a sizeable crucian
399 carp population (CPUE = 60.50), but samples from CHIP were not inhibited. Crucian carp
400 DNA was not detected at any sites where the species was absent.

401

402

403 **3.4 Factors influencing eDNA detection and quantification**

404

405 The occupancy model with the best support included CPUE and conductivity as covariates of
406 eDNA detection probability in qPCR replicates (p). The model did not include any covariates
407 of eDNA occurrence probability at sites (ψ) or eDNA detection probability in water samples
408 (θ). Estimates of eDNA detection probability in a qPCR replicate ranged between 0.12 to
409 1.00 (Table 1), where crucian carp CPUE (parameter estimate = 1.357) and conductivity
410 (parameter estimate = -2.112) played positive and negative roles in eDNA availability
411 respectively (Figures 4a, b). The GLMM identified CPUE (0.020 ± 0.007 , $\chi^2_1 = 5.426$, $P =$
412 0.020) and conductivity (-0.007 ± 0.002 , $\chi^2_1 = 8.709$, $P = 0.003$) as significant predictors of
413 DNA copy number, where DNA copy number was greater at higher CPUE (Figure 5a) but
414 decreased as conductivity increased (Figure 5b).

415

416

417

418 **4. Discussion**

419

420 We developed a novel species-specific qPCR assay to enable large-scale distribution
421 monitoring of the threatened crucian carp using eDNA. Crucian carp were detected at almost
422 all sites with confirmed presence and no false positives were generated. Furthermore, biotic

423 and abiotic factors that influence eDNA detection and quantification were identified. We
424 discuss areas for improvement in our workflow and provide recommendations for future
425 study.

426

427

428 **4.1 Using eDNA for crucian carp conservation**

429

430 eDNA analysis is often compared to conventional monitoring tools to assess performance,
431 reliability, reproducibility, and prospective applications in conservation programmes. We
432 found strong agreement between eDNA and fyke netting for crucian carp detection, where
433 eDNA detected crucian carp in 90% of ponds with presence confirmed by netting. This high
434 detection and low false negative rate supports the applicability of eDNA analysis to crucian
435 carp presence-absence monitoring, particularly at large spatial scales where fyke netting can
436 be costly and time-consuming, and where ponds are remote with poor access. Abundance
437 estimation is less straightforward as there was uncertainty around the relationship between
438 DNA copy number and crucian carp density. This inconsistency is more likely to result from
439 eDNA than fyke netting due to effects exerted by external factors (section 4.2) and sample
440 processing (section 4.3) on eDNA quality. However, fyke netting also has detection biases
441 that may influence performance comparisons with eDNA. Fyke net surveys are restricted
442 spatially and temporally to pre- and post-spawning as well as spring and autumn when
443 temperatures are low to reduce fish stress in nets. Furthermore, fyke net surveys may fail to
444 capture species that do not have homogenous distribution in their environment, especially
445 where populations contain few individuals (Turner et al., 2012). Netting is also biased
446 towards particular fish size classes that can enter nets through standard European otter (*Lutra*
447 *lutra*) guards (75 mm in UK), and catchability is further dependent on time of year (Ruane,

448 Davenport & Igoe, 2012) and even time of day (Hardie, Barmuta & White, 2006). Therefore,
449 effectiveness of standard methods must also be evaluated and eDNA compared to multiple
450 tools before deemed capable or incapable of estimating abundance.

451

452

453 **4.2 Factors influencing eDNA detection and quantification**

454

455 Effects of biotic and abiotic factors on eDNA may vary across target species and ecosystems
456 (Barnes et al., 2014). We found crucian carp density (CPUE) positively influenced eDNA
457 detection probability and DNA copy number. Density is frequently reported to improve
458 detection probability of aquatic species due to more eDNA deposition in the environment
459 (Schmelzle & Kinziger, 2016; Buxton et al., 2017b; Stoeckle et al., 2017), but this
460 relationship is highly variable across study systems and species due to influence of external
461 factors (Strickler et al., 2015; Buxton et al., 2017a; Goldberg et al., 2018). For example,
462 increase in water temperature coincided with breeding activity and heightened DNA release
463 in other fish and amphibian species (Buxton et al., 2017b; Bylemans et al., 2017). In our
464 study, CPUE was collinear with water temperature and thus water temperature was not
465 included in our occupancy model or GLMM. We performed water sample collection in late
466 August, which is outside the reported spawning period for crucian carp (Aho & Holopainen,
467 2000). However, the association between CPUE and DNA copy number may be linked to
468 increased DNA shedding rates caused by higher metabolic activity in response to warm
469 temperature, as reported for other fish species (Takahara et al., 2012; Robson et al., 2016;
470 Lacoursière-Roussel et al., 2016).

471 In contrast to CPUE, conductivity had a negative effect on eDNA detection and
472 concentration. Conductivity has been suggested to influence eDNA detection and

473 quantification, but studies that directly measured this variable have found no discernable
474 effect (Takahara et al., 2012; Keskin, 2014; Goldberg et al., 2018). Conductivity (also
475 measured as TDS) relates to sediment type which influences eDNA detection probability, the
476 rate at which sediment binds eDNA, and release of inhibitory substances (Buxton et al.,
477 2017a; Stoeckle et al., 2017). Notably, the only false negative pond in our study was also the
478 most conductive (760 $\mu\text{s}/\text{cm}$) and possessed dense beds of rigid hornwort (*Ceratophyllum*
479 *demersum*) that could restrict water movement. Therefore, conductivity may lead to incorrect
480 inferences about species presence and impact conservation management decisions. Further
481 investigation into the effects of conductivity on eDNA detection and quantification is clearly
482 needed.

483 Our results indicate that samples may have been affected by inhibitory substances
484 despite tests performed to identify inhibition. We spiked qPCR reactions with a known
485 amount of synthetic target DNA; however, an artificial Internal Positive Control gene assay
486 may identify inhibition more effectively (Goldberg et al., 2016). Dilution of eDNA samples
487 (and inhibitory substances present) can release inhibition, but also reduce detection
488 probability (Piggott, 2016) and induce false negatives (Buxton et al., 2017a). We used
489 TaqMan[®] Environmental Master Mix 2.0 (Life Technologies, CA, USA) in qPCR reactions
490 to counter inhibition (Jane et al., 2015), but it may be advisable to use DNA extraction kits
491 that perform inhibitor removal (Sellers et al., 2018) or include Bovine-serum albumin (BSA)
492 in qPCR reactions (Jane et al., 2015). Alternatively, ddPCR may handle inhibitors better than
493 qPCR and provide more accurate abundance or biomass estimates (Nathan et al., 2014).

494 Crucially, environmental data were not collected in 2016 for every pond in our study.
495 Our results indicate direction of effects of biotic and abiotic factors on eDNA detection and
496 quantification, but contemporary data are needed for comprehensive interpretation of these
497 relationships. However, it is clear that eDNA practitioners must account for these effects as

498 well as sample inhibition. The uncertainty around the estimated effects of covariates in our
499 hierarchical occupancy model and GLMM also imply that greater sample volume, sample
500 number, and/or qPCR replication are required to improve the ability and precision of our
501 assay to detect crucian carp eDNA and reduce the potential for false negatives (Schultz &
502 Lance, 2015; Goldberg et al., 2018).

503

504

505 **4.3 Optimisation of eDNA workflow**

506

507 Some non-target DNA extracts used to validate assay specificity were contaminated with
508 crucian carp DNA. Field cross-contamination can occur if reference tissue material is
509 collected from multiple species without sterilising equipment, or eDNA is present on the
510 material collected (Rodgers, 2017). Collection and storage of reference tissue material is an
511 important consideration for eDNA practitioners, particularly those using highly sensitive
512 assays (LOD = 1 copy/ μ L) (Wilcox et al., 2013, 2016). Dedicated, sterilised equipment
513 should be used when collecting new reference material from different species. From existing
514 reference collections, only non-target samples that were collected on separate and
515 chronologically distinct occasions from target samples should be used (Rodgers, 2017).

516 Cross-contamination can also arise during water sampling, filtration, DNA extraction
517 and qPCR preparation. Low-level contamination was found in one full process blank but
518 detections from this pond were not omitted as it contained crucian carp and contamination
519 was not observed downstream. All equipment in our study was sterilised by immersion in 10%
520 chlorine-based commercial bleach (Elliott Hygiene Ltd, UK) solution for 10 mins, followed
521 by rinsing in 5% MicroSol detergent (Anachem, UK), and then purified water. However,
522 sterilisation with 50% chlorine-based commercial bleach solution (Goldberg et al., 2016) or

523 single-use, sterile materials (Wilcox et al., 2016) may further minimise contamination risk.

524 Many of our eDNA samples were low concentration (<100 copies/ μ L) which can
525 cause inconsistent qPCR amplification (Goldberg et al., 2016), thus we discuss approaches to
526 maximise eDNA concentration and improve detection probability. The probability of eDNA
527 detection depends heavily on the number of samples and volume of water collected, time of
528 sampling, and sample concentration (Schultz & Lance, 2015; Goldberg et al., 2018). We
529 sampled 5 x 2 L water samples from each pond in autumn 2016, but timing and/or sampling
530 effort may have been inappropriate. A seasonal effect on common carp eDNA detection was
531 observed, where spring sampling generated higher eDNA concentration and detection rates
532 due to greater common carp activity (Turner et al., 2014) and density (Hinlo et al., 2017a). As
533 water sampling did not coincide with fyke netting (spring 2016) in our study, eDNA
534 concentration may not reflect CPUE estimates. Water samples in spring may contain more
535 crucian carp eDNA due to higher activity of individuals, or autumn fyke netting may produce
536 lower CPUE estimates. Parallel seasonal sampling, where water sampling is performed in
537 conjunction with fyke netting at different times of the year, may better align eDNA
538 concentration with CPUE estimates and enable eDNA-based abundance estimates for crucian
539 carp. This is certainly a worthwhile area of research.

540 Representative sampling is crucial in eDNA surveys. Individuals of a species can be
541 unevenly distributed in the environment, which impacts eDNA detection, distribution, and
542 concentration (Takahara et al., 2012; Eichmiller, Bajer & Sorensen, 2014; Schmelzle &
543 Kinziger, 2016; Goldberg et al., 2018). In lentic ecosystems, eDNA has a patchy horizontal
544 and sometimes vertical distribution, resulting in fine spatial variation (Eichmiller et al., 2014).
545 Studies on common carp revealed eDNA was more concentrated near the shoreline of lentic
546 water bodies (Takahara et al., 2012; Eichmiller et al., 2014), due to aggregations of
547 individuals (Eichmiller et al., 2014). We collected surface water (5 x 2 L) from the shoreline

548 and sampled at equidistant points around the pond perimeter where possible; however, more
549 samples and greater water volumes may be required to improve detection probability (Schultz
550 & Lance, 2015; Goldberg et al., 2018). Fine spatial sampling and occupancy modelling are
551 needed to determine the sample number required to achieve high detection probability and
552 eliminate false negatives (Goldberg et al., 2018). However, the number of samples required
553 will inevitably vary across habitats due to inherently variable physical properties (Schmelzle
554 & Kinziger, 2016).

555 Method of eDNA capture can dictate success of this monitoring tool. Studies of
556 eDNA in ponds (Ficetola et al., 2008; Biggs et al., 2015) have used an ethanol precipitation
557 approach, but this is restricted to small volumes. Filtration allows more water to be processed
558 and minimises capture of non-target DNA, with macro-organism eDNA effectively captured
559 by pore sizes of 1 - 10 μm (Turner et al., 2014). We used a small pore size of 0.45 μm to
560 capture most eDNA particle sizes, although filter clogging prevented the entire sample being
561 processed and may have affected eDNA concentration downstream. Pre-filtering can reduce
562 clogging, but is labour-intensive and increases cost (Takahara et al., 2012). Larger pore sizes
563 have been used in temperate and tropical lentic environments (Takahara et al., 2012; Robson
564 et al., 2016; Goldberg et al., 2018), though independent investigation is needed to determine
565 which pore size maximises target DNA concentration.

566 Comparisons of eDNA yield across filter types and DNA extraction protocols have
567 shown that cellulose nitrate filters stored at -20 °C (this study) often provide best eDNA yield
568 (Piggott, 2016; Spens et al., 2016; Hinlo et al., 2017b). However, different filter types may be
569 optimal for different species, which has consequences for detectability (Spens et al., 2016)
570 and relationships between eDNA concentration and abundance/biomass (Lacoursière-Roussel
571 et al., 2016). Extraction method used, regardless of filter type, will ultimately influence DNA
572 quality and yield. We used the PowerWater[®] DNA Isolation Kit (MO BIO Laboratories, CA,

573 USA), but the DNeasy Blood and Tissue kit (Qiagen[®], Hilden, Germany) has demonstrated
574 greater yield (Hinlo et al., 2017b). We also combined filters from the same sample for DNA
575 extraction at the bead milling stage, but independent lysis may recover more DNA (Hinlo et
576 al., 2017b). A comparison of DNA extraction protocols is necessary to assess which approach
577 maximises crucian carp eDNA concentration. A new modular extraction method shows
578 promise for eDNA but has yet to be evaluated for targeted qPCR (Sellars et al., 2018).

579 Finally, detection sensitivity can be enhanced by increasing the number of qPCR
580 technical replicates (Schultz & Lance, 2015; Piggott, 2016). We performed five technical
581 replicates for each of our samples, but other studies have used as many as twelve and only
582 one may amplify (Biggs et al., 2015). More replication may have enabled amplification from
583 the CHIP pond samples, but qPCR cost would inevitably increase. Further replication may
584 also be unnecessary if steps are taken to improve initial concentration of samples instead
585 (Schultz & Lance, 2015).

586

587

588 **4.4 Concluding remarks**

589

590 A primary objective of the Norfolk crucian carp BAP was to obtain a basic understanding of
591 species distribution and population status across Norfolk (Copp & Sayer, 2010). eDNA
592 surveillance for crucian carp will provide a useful, cost-effective alternative to established
593 survey methods where the aim is determining presence-absence. Our assay may detect
594 hybrids where crucian carp were the maternal parent due to use of a mitochondrial marker;
595 however, these detections are also beneficial to the crucian carp conservation effort through
596 the identification of ponds where true crucian carp may still exist, and where contamination
597 with goldfish, common carp and their hybrids has occurred. Alternatively, our assay could be

598 used as an early warning tool in countries where the crucian carp is considered invasive. The
599 areas we have highlighted require further investigation before eDNA can be used routinely.
600 Nevertheless, eDNA survey could enable large-scale distribution monitoring for crucian carp
601 through rapid screening of existing and new ponds. Fyke netting could then be used to
602 investigate age, sex and size structure of populations, and remove hybrids.

603

604

605

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607

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613

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615

616 **Conflict of interest**

617

618 The authors declare no conflict of interest.

619

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837

838 **Table 1.** Bayesian estimates of crucian carp eDNA occurrence probability at a pond (ψ), eDNA detection
839 probability in a water sample (θ), and eDNA detection probability in a qPCR replicate (p). Posterior median and
840 95% credible interval (CI) are given for each parameter of the occupancy model. The corresponding catch-per-
841 unit-effort estimate (CPUE) is given for each pond.

842

Pond	Crucian carp (Y/N)	CPUE estimate	ψ		θ		p	
			Posterior median	95% CI	Posterior median	95% CI	Posterior median	95% CI
CAKE	Y	43.00	0.90	0.62 – 1.00	0.83	0.70 - 0.92	0.14	0.05 - 0.33
CHIP	Y	60.50	0.90	0.62 – 1.00	0.83	0.70 - 0.92	0.12	0.03 - 0.36
GUES1	Y	121.75	0.90	0.62 – 1.00	0.83	0.70 - 0.92	0.98	0.86 - 1.00
MYST	Y	6.17	0.90	0.62 – 1.00	0.83	0.70 - 0.92	0.93	0.86 - 0.98
OTOM	Y	14.67	0.90	0.62 – 1.00	0.83	0.70 - 0.92	0.96	0.91 - 0.99
POFA4	Y	13.67	0.90	0.62 – 1.00	0.83	0.70 - 0.92	0.89	0.81 - 0.95
POHI	Y	7.25	0.90	0.62 – 1.00	0.83	0.70 - 0.92	0.44	0.28 - 0.59
RAIL	Y	58.17	0.90	0.62 – 1.00	0.83	0.70 - 0.92	1.00	0.99 - 1.00
SKEY1	Y	31.38	0.90	0.62 – 1.00	0.83	0.70 - 0.92	1.00	1.00 - 1.00
WADD3	Y	126.00	0.90	0.62 – 1.00	0.83	0.70 - 0.92	1.00	1.00 - 1.00

843

844 **Table 2.** Top NCBI BLASTn hit for Sanger sequences obtained from target DNA (tissue extracts and synthetic
845 gBlocks® Gene Fragment), non-target tissue DNA extracts, full process blanks, and representative eDNA
846 samples that amplified during qPCR. Sample descriptions marked with ‘!’ indicate a poor quality, discarded
847 sequence.

848

Sample	Description	Query length	Coverage	E value	Identity	Accession
CrucianCarp-01	<i>Carassius carassius</i>	37	100%	3E-09	100%	KR131843.1
CrucianCarp-02	<i>Carassius carassius</i>	37	100%	3E-09	100%	KR131843.1
CrucianCarp-03	!					
Gblock-100copies-01	<i>Carassius carassius</i>	37	100%	3E-09	100%	KR131843.1
Gblock-100copies-02	!					
Gblock-100copies-03	!					
Rudd-JL-01	<i>Carassius carassius</i>	38	100%	9E-10	100%	KR131843.1
Rudd-JL-02	<i>Carassius carassius</i>	38	100%	9E-10	100%	KR131843.1
Rudd-JL-03	!					
Rudd-PS-01	!					
Rudd-PS-02	!					
Rudd-PS-03	!					
Chub-PS-01	!					
Chub-PS-02	!					
Chub-PS-03	!					
Chub-JL-01	!					
Chub-JL-02	!					
Chub-JL-03	!					
CommonCarp-01	!					
CommonCarp-02	!					
CommonCarp-03	!					
POFA4-B-01	!					
POFA4-B-02	!					

POFA4-B-03	!					
GUES1-5-01	<i>Carassius carassius</i>	37	100%	3E-09	100%	KR131843.1
GUES1-5-02	<i>Carassius carassius</i>	41	100%	1E-07	95%	KR131843.1
GUES1-5-03	<i>Carassius carassius</i>	41	100%	2E-11	100%	KR131843.1
MYST-3-01	<i>Carassius carassius</i>	46	100%	4E-14	100%	KR131843.1
MYST-3-02	!					
MYST-3-03	<i>Carassius carassius</i>	41	100%	1E-07	95%	KR131843.1
SKEY1-4-01	<i>Carassius carassius</i>	35	100%	4E-08	100%	KR131843.1
SKEY1-4-02	!					
SKEY1-4-03	<i>Carassius carassius</i>	37	100%	3E-09	100%	KR131843.1
OTOM-4-01	!					
OTOM-4-02	!					
OTOM-4-03	<i>Carassius carassius</i>	37	100%	3E-09	100%	KR131843.1
POHI-2-01	<i>Carassius carassius</i>	41	100%	2E-11	100%	KR131843.1
POHI-2-02	!					
POHI-2-03	<i>Carassius carassius</i>	37	100%	3E-09	100%	KR131843.1
RAIL-4-01	!					
RAIL-4-02	<i>Carassius carassius</i>	38	100%	9E-10	100%	KR131843.1
RAIL-4-03	<i>Carassius carassius</i>	46	100%	4E-14	100%	KR131843.1
WADD3-4-01	<i>Carassius carassius</i>	25	96%	0.034	100%	KR131843.1
WADD3-4-02	!					
WADD3-4-03	<i>Carassius carassius</i>	38	100%	9E-10	100%	KR131843.1
CAKE-1-01	!					
CAKE-1-02	!					
CAKE-1-03	!					
POFA4-5-01	!					
POFA4-5-01	!					
POFA4-5-01	!					

850 **Figure 1.** A crucian carp (*Carassius carassius*) individual (**a**) and examples of the study ponds (**b-d**). Photo (**a**)
851 by John Bailey and photo (**d**) by Sacha Dench.

852

853 **Figure 2.** Map of pond locations in North Norfolk, eastern England, showing the distribution of ponds
854 containing crucian carp (black dots) and ponds where the species is absent (grey dots).

855

856 **Figure 3.** Alignment of consensus sequences for a region of the mitochondrial cytochrome *b* (*cytb*) gene in 24
857 European freshwater fishes, including the crucian carp. Species-specific primers and probe for the crucian carp
858 are given on the first line. Consensus with primer and probe sequence across species is highlighted in white
859 whereas mismatches are coloured by nucleotide base.

860

861 **Figure 4.** Estimated probability of eDNA detection in qPCR replicates. Points are estimates of posterior
862 medians with 95% credible intervals. Probability of eDNA detection in qPCR replicates increased with higher
863 catch-per-unit-effort (CPUE) estimate (**a**) but decreased as conductivity increased (**b**).

864

865 **Figure 5.** Relationship between fixed effects and response variable (DNA copy number) in ponds, as predicted
866 by the Poisson GLMM. The 95% CIs, as calculated using the model predictions and standard error for these
867 predictions, are given for each relationship. The observed data (points) are also displayed against the predicted
868 relationships (line). DNA copy number increased with catch-per-unit-effort (CPUE) estimate (**a**), but decreased
869 as conductivity (**b**) increased.

870